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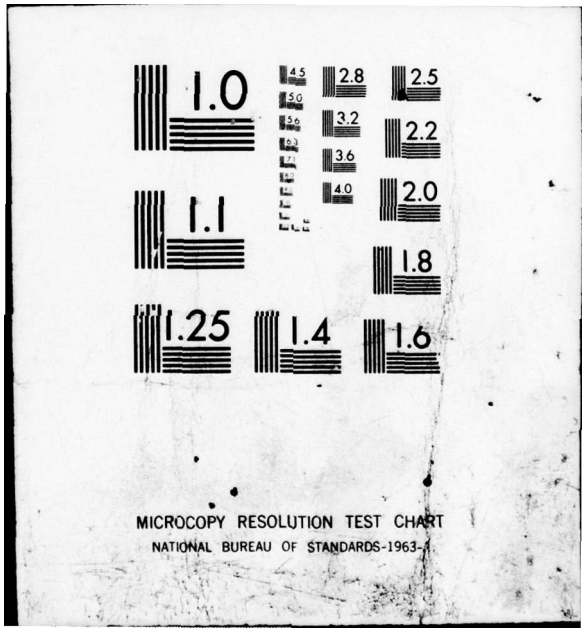
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DEVELOPMENT OF METHODS FOR NEURORRHAPHY AND THE TREATMENT OF SPINAL CORD AND CAUDA EQUINA INJURIES IN BATTLE CASUALTIES

Final Report

August 1978

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by

Joseph Ransohoff, M.D.

Supported by

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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

The role of free radical lipid peroxidation in spinal cord injury has been studied. A potentiating effect of ethanol was observed in both acute and chronic models of spinal cord injury. Lipid peroxidation following spinal cord injury was determined by measuring lipid soluble fluorescence, found to be higher in the ethanol-treated animals than in the controls and non-ethanol treated animals. Attempts at treatment were made using free radical scavengers. DMSO and mannitol were selected. No improvement was seen with mannitol. Seven

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of 20 animals receiving DMSO improved in neurologic function.

Studies on the role of catecholamines in spinal cord injury were extended to turnover studies. These demonstrated alterations in the membrane-bound enzymes, dopamine-beta-hydroxylase and monoamine oxidase. It is proposed that the damage to membranes may be mediated by free radical mechanisms and that the alterations in catecholamine metabolism seen may be due to inhibition of membrane-bound enzymes following free radical damage to the membrane itself.

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FINAL SCIENTIFIC REPORT

Department of the Army
U.S. Army Medical Research and Development Command

Contract Number DADA17-73-C-3021

Narrative Summary

A major effort was the study of the potentiation of central nervous system (CNS) trauma by ethanol. This was undertaken because of the known effect of ethanol on free radical lipid peroxidation, as well as the frequent clinical association of alcohol with trauma to the central nervous system, both spinal cord injury and head injury. Two models were used to study this. The spinal cord contusion model was used in both acute and chronic animals. In the acute animals, a 200 gm-cm force failed to produce loss of evoked potentials. However, in animals pretreated with ethanol to achieve a blood level of 300 mg % prior to the injury, evoked potentials following a 200 gm-cm force were invariably lost. In a chronic study, those animals pretreated with alcohol remained permanently paraplegic, whereas those who had received a 200 gm-cm force, but no alcohol, regained their ability to walk. The effects of alcohol were studied in a model of cerebral contusion. A 900 gm-cm force was delivered to the exposed dura over one cerebral hemisphere. All animals received Evans blue prior to the injury. Half of the animals also received an infusion of ethanol to achieve the same blood levels as the spinal cord injured animals. The remaining animals received no ethanol. The extent of the injury was 3-4 times as large in the alcohol pretreated group. This material was published in the Journal of Neurosurgery (Flamm, E.S., Demopoulos, H.B., Seligman, M.L., Tomasula, J.J., DeCrescito, V. and Ransohoff, J.: Ethanol potentiation of central nervous system trauma. J. Neurosurg. 46: 328-335, 1977). The implications of these observations are that ethanol or its metabolites, particularly acetaldehyde, may potentiate the effect of the injury through free radical mechanisms. Other explanations may rest in the changes in clotting mechanisms produced by this level of alcohol. Because our previous attempts to measure malonaldehyde as an indicator of free radical damage were not totally satisfactory, we have also used this model to study the presence of malonaldehyde and other reactions which it undergoes such as the formation of Schiff base products. This was carried out by spectrophotofluorometry. This study showed a rise in lipid soluble fluorescence in both the control animals and alcohol-treated animals at 3 days following their injury. This peak returned to baseline levels at 5 days following injury and at 7 days, it rose again in only the alcohol pretreated group, thus indicating that malonaldehyde and its addition products are certainly present at a later stage following injury and are increased by the presence of alcohol. Further study is required to determine if free radical mechanisms are operative at the very early post-injury stages. These data were published in Lipids (Seligman, M.L., Flamm, E.S., Goldstein, B.D., Poser, R.G., Demopoulos, H.B. and Ransohoff, J.: Spectrofluorescent detection of malonaldehyde as a measure of lipid free radical damage in response to ethanol potentiation of spinal cord trauma. Lipids 12: 945-950, 1977).

Several agents known to have the potential for trapping free radicals were used in a therapeutic trial. A control group and two treatment groups, one which received Mannitol and one which received dimethyl sulfoxide (DMSO), were studied following a 400 gm-cm impact. Each animal received 1.5 gm/kg in several dosage schedules. This study was carried out as a double-blind study. No improvement was seen in the group treated with Mannitol following injury. In the DMSO group, 7 out of 20 animals showed return of neurologic function.

To define better our preliminary studies on catecholamine changes following spinal cord injury, turnover studies were carried out using tritiated tyrosine and L-dopa. These studies demonstrated a reduction in activity of tyrosine hydroxylase as evidenced by lower amounts of tritiated dopamine formed from both the tyrosine and L-dopa precursors. Furthermore, activity of membrane-bound enzymes, dopamine beta hydroxylase and mitachondrial monoamine oxidase, was curtailed. This resulted in an accumulation of dopamine and no change in norepinephrine concentration. These results would indicate that those changes of catecholamine metabolism seen following spinal cord injury probably reflect membrane perturbations and disruption of function of membrane-bound enzymes; thus, catecholamine alterations are probably a secondary phenomena and do not play an etiologic role in the development of degenerative changes following spinal cord injury. This work was published in a book, Catecholamines and Stress (Naftchi, N.E., Demeny, M., Flamm, E.S. and Lowman, E.W.: Effect of traumatic stress on catecholamine synthesis and metabolism in the spinal cord. IN: Catecholamines and Stress, Usdin, E. (ed.), New York: Pergamon Press, 1976, pp. 367-375.).

The methodology for analysis of central nervous system tissue by gas liquid chromatography has been developed and profiles of changes in long-chain fatty acids are beginning to be obtained. This work will be continued.

Since our observation that the epr signal for ascorbic acid is decreased following insult to the central nervous system, either by trauma to the spinal cord or the production of cerebral ischemia, we have been interested in the significance of these findings in relation to ascorbic acid's role as a free radical scavenger and antioxidant. In a model of artificial membrane, using liposomes in which peroxidation was induced by exposure to U.V. light, the addition of ascorbic acid was effective in reducing the extent of peroxidation measured by malonaldehyde determinations. Furthermore, the epr signal for ascorbic acid was reduced in the presence of peroxidized liposomes. This in vitro model correlates well with the results obtained in the spinal cord injured animal.

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Ethanol potentiation of central nervous system trauma

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✔ Two models have been used to study the effects of ethanol on injuries of the central nervous system. The spinal cords of cats were injured by delivering a 200 gm-cm impact to the exposed dura mater. A second group of animals received a similar injury to the exposed dura mater overlying the cerebral hemispheres. The animals were divided into two groups, those that received an infusion of ethanol before injury, and control animals that received no ethanol. The parameters of injury used in this model produced small and insignificant lesions in those animals that received no ethanol; however, when the animals were pretreated with ethanol, a considerable increase in the extent of the injury was noted. The mechanisms by which this potentiation is produced are briefly discussed. These include alterations in membrane-bound enzymes and clotting mechanisms, and alteration of cell membranes through abnormal free radical reactions.

KEY WORDS • **spinal cord injury** • **cerebral contusion** • **ethanol** • **lipid peroxidation** • **trauma** • **free radical damage** • **alcohol**

A GROWING body of evidence indicates that moderate ethanol intake, even in the presence of adequate nutrition, may result in cell injury and cell death in the liver, myocardium, and central nervous system (CNS). Ethanol, its principal metabolite, acetaldehyde, and the intermediaries, which are free radicals, possess several different chemical properties that have the potential for many adverse reactions in a cell. The lipophilic property of ethanol results in its intercalation into cell membranes. In such key regulatory locations, alcohol may affect the critical relationships among the membrane biomolecules by physical forces or chemical reactions, and consequently cause cellular dysfunction and cell death.

Among the chemical abnormalities that can be induced by ethyl alcohol are a group known as free radical reactions. This has been demonstrated in the lipids of mitochondrial and other cell membranes in the hepatocytes of experimental animals.[11,12] A free radical is a reactive species characterized by an unpaired electron. This allows the molecule to enter into many reactions that do not occur with its stable non-radical precursor.[31] The occurrence of such reactions among the lipids that make up the extensive membrane systems in the CNS might explain, at least in part, some of the toxic effects of ethanol.

Alcohol and acute CNS trauma are frequently associated together in the clinical setting, and it is possible that there is also a relationship at cellular and membrane levels. This

laboratory has long been interested in the role of free radical damage to the membrane lipids of the CNS in posttraumatic degeneration.[7-9,23,25] In previous studies, evidence has been obtained to support the hypothesis of free radical destruction of lipids following trauma to the cat spinal cord.[23]

To delineate further the role of radical damage to membrane lipids in the CNS, the question was posed whether CNS trauma, known to produce some changes compatible with this molecular change, could be enhanced by ethanol. Ethyl alcohol was selected because of its frequent association with trauma in clinical situations, and because of its induction of free radical lipid damage in other tissues.[11,12,26,27]

Two models were employed for this study. One was a spinal cord injury produced by dropping a weight on the exposed dural tube, and the other a model of cerebral contusion produced by dropping a weight on the exposed dura over the cerebral hemisphere. In both studies, animals received alcohol before injury, and the results were compared with animals injured without alcohol.

Materials and Methods

We used 165 adult cats, ranging in weight from 2.7 to 3.5 kg. All animals were anesthetized with sodium pentobarbital, 30 mg/kg, and allowed to breathe spontaneously; body temperature was maintained with a heating blanket. Arterial blood pressure and blood gases were monitored. The spinal cord injury series comprised 93 cats, and 72 cats had cerebral contusion. Blood alcohol levels were measured spectrophotometrically using a dichromate assay.[24]

Spinal cord injury was produced according to a method previously reported from this laboratory.[2,3,15] A laminectomy was carried out from T-7 through T-11. A 20-gm weight was dropped 10 cm down a Teflon tube to strike a 2.4-gm impactor with a diameter of 0.5 cm, thereby delivering a 200 gm-cm force injury. Before laminectomy, after laminectomy, and periodically after injury, evoked potentials were monitored according to techniques described previously.[3] Fifty-six cats received 95% ethanol, 5 ml/kg, diluted with saline to a volume of 30 ml, as a constant infusion for 1 hour prior to laminectomy or injury. An injury to the spinal cord was delivered in 37 cats without any ethanol infusion. Table 1 shows the distribution of the 93 cats in the spinal cord study. Acute studies were performed in 66 animals which were sacrificed at 3, 5, and 24 hours after injury. The 27 chronic animals were followed with frequent neurological examinations and repeat evaluation of evoked potentials. At the time of sacrifice, all animals were perfused with 10% formalin. A 5-cm segment of cord, centered at the site of injury, was removed and sectioned serially. The sections were stained with hematoxylin and eosin as well as Luxol fast blue-PAS.

TABLE 1

Course in 93 cats that underwent spinal cord injury

Procedure*	Time of Sacrifice†			
	3 hrs	5 hrs	24 hrs	6–9 wks
ethanol + laminectomy	6	4	8	
ethanol + 200 gm-cm impact	12	7	5	14
200 gm-cm impact	12	7	5	13

*Ethanol was administered as an infusion of 95% ethanol, 5 ml/kg, diluted with saline to a volume of 30 ml, as a constant infusion for 1 hour before laminectomy or injury.

†Time after laminectomy or injury.

In the second injury model, 72 cats underwent cerebral contusion.[6] A left frontoparietal craniectomy was performed with a trephine. The same apparatus used in the spinal cord injury was used to deliver an injury of 200 to 1000 gm-cm impact to the exposed dura over the cerebral hemisphere. All animals received an injection of 5 ml of Evans blue before injury. An infusion of 95% ethanol, 5 ml/kg, diluted with saline to a volume of 30 ml, was given to 44 cats for 1 hour before injury.

Preliminary studies showed no cerebral lesion when a 200 to 600 gm-cm force was delivered. For this reason, the major portion of the study was carried out using an 800 to 1000 gm-cm force. Of 21 animals that received an 800 gm-cm impact, 13 were pretreated with an infusion of ethanol, and six served as controls. Of 26 cats that received a 900 gm-cm impact, 15 were pretreated with ethanol. A 1000 gm-cm impact was administered to 19 animals, 12 of which received an infusion of ethanol before impact.

TABLE 2

Course in 72 cats that underwent cerebral contusion

Procedure	3-Hr Study		24-Hr Study	
	Ethanol*	Control	Ethanol*	Control
800 gm-cm impact	9	3	4	3
900 gm-cm impact	10	6	5	5
1000 gm-cm impact	7	3	5	4

*Ethanol was administered as an infusion of 95% ethanol, 5 ml/kg, diluted with saline to a volume of 30 ml, as a constant infusion for 1 hour before laminectomy or injury.

The distribution of the animals into different groups is shown in Table 2. At the time of sacrifice, all animals were perfused with 10% formalin; the brains were removed and sectioned coronally. Photographs of the gross specimens and histological studies were obtained.

Results

No significant variations were seen in blood pressure and heart rate when ethanol was administered. Blood gases remained within normal limits. Blood ethanol values were obtained after 15, 30, 45, and 60 minutes of infusion, and then at 1, 2, 3, and 24 hours after impact. After 30 minutes of infusion, a mean value of 199 mg% was obtained. This rose to 448 mg% after 60 minutes of infusion which was the time of injury. Thereafter, the blood alcohol levels fell to 360 mg% by 2 hours. At 3 hours, a mean blood alcohol level of 119 mg% was observed. Blood alcohol was at undetectable levels by 24 hours after the infusion and trauma.

Spinal Cord Studies

The 18 animals that received ethanol before laminectomy, but no injury to the cord, maintained their evoked potentials throughout the study. In the 24 animals that received ethanol followed by a 200 gm-cm force impact, the evoked potentials disappeared immediately after the injury and showed no sign of return in the next 3 hours. The 24 animals that received a 200 gm-cm impact without any ethanol infusion retained their evoked potentials after the injury in all but two animals.

In the histological studies, the ethanol-laminectomy group showed no abnormalities. Six of the 24 animals that received a 200 gm-cm injury without ethanol had small areas of hemorrhage within the gray matter (Fig. 1 *left*). The spinal cords of the remaining 18 animals were histologically normal. In the ethanol-impact group, extensive changes were seen following the 200 gm-cm injury (Fig. 1 *right*). These included hemorrhage in the gray matter spreading out into the white matter and, in the 5-hour and 24-hour groups, a considerable amount of edema and hemorrhage in the white matter. These spinal cords appeared identical to those seen in this laboratory following a 400 gm-cm injury at the same time intervals.[3]

The long-term study involved 27 cats. The 13 animals that received the 200 gm-cm impact without ethanol all retained their evoked potentials during the next 24 hours. They were followed clinically for 6 to 9 weeks. By the end of 5 days, all animals were walking, although three were considered poor walkers even after 9 weeks (Fig. 2). Of the 14 animals treated with ethanol before injury, the evoked response was abolished in 12, but retained in two. These animals were followed from 6 to 9 weeks (Fig. 3). During this time, 12 animals remained totally paraplegic. The two animals in which the evoked potential was preserved regained their ability to walk. One walked well and the other was considered a poor walker.

Cerebral Contusion Study

The eight animals that received a 200 to 600 gm-cm impact to the cerebral hemisphere showed no lesions as judged by extravasation of Evans blue dye or by histological examination. In the 19 animals that received an 800 gm-cm impact, 12 were examined at 3 hours and seven at 24 hours. A small area of extravasation of Evans blue was seen in the white matter immediately beneath the area of impact. No differences were seen between the animals that received ethanol and those that did not, either at 3 hours or at 24 hours. When a 900 gm-cm force was used, distinct differences were seen between animals that received alcohol and those that did not. This group included 26 animals, 16 studied at 3 hours and 10 at 24 hours. All animals showed

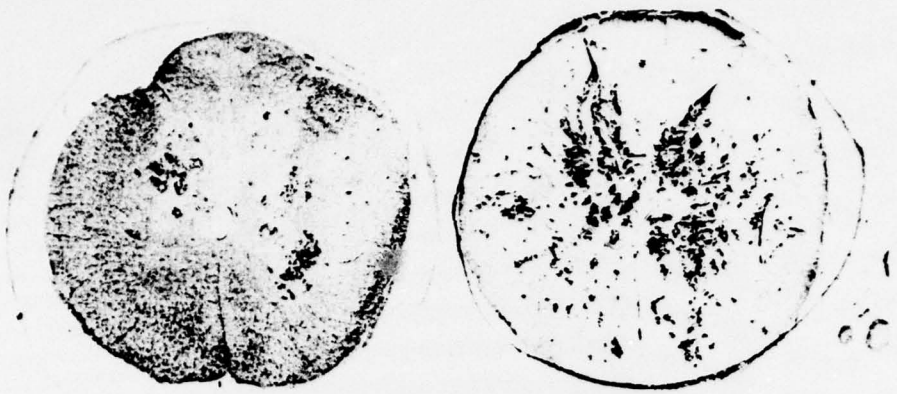

FIG. 1. Cat spinal cords at T-9. H & E, × 2. *Left:* At 3 hours after 200 gm-cm impact injury. Small areas of hemorrhage are present throughout the gray matter. *Right:* At 3 hours after 200 gm-cm impact injury and ethanol infusion. Extensive hemorrhage in gray matter.

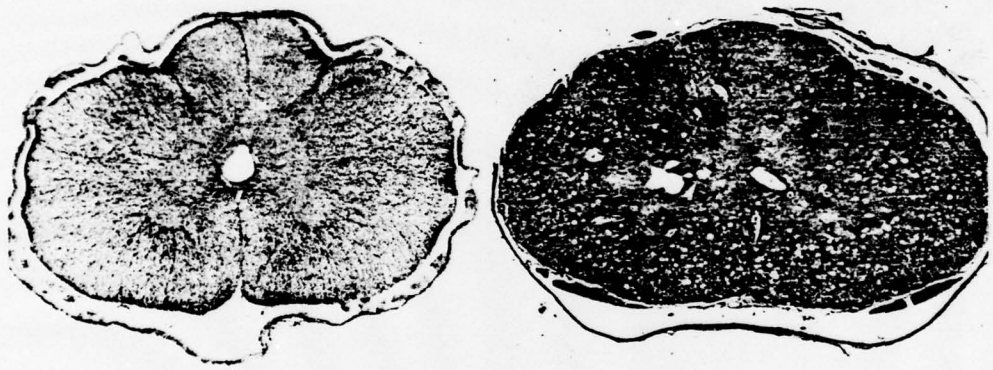

FIG. 2. Cat spinal cords at T-9. H & E, × 2. *Left:* At 7 weeks after 200 gm-cm impact injury. *Right:* At 8 weeks after 200 gm-cm impact. These two sections show the range of changes seen in the 13 animals of this group.

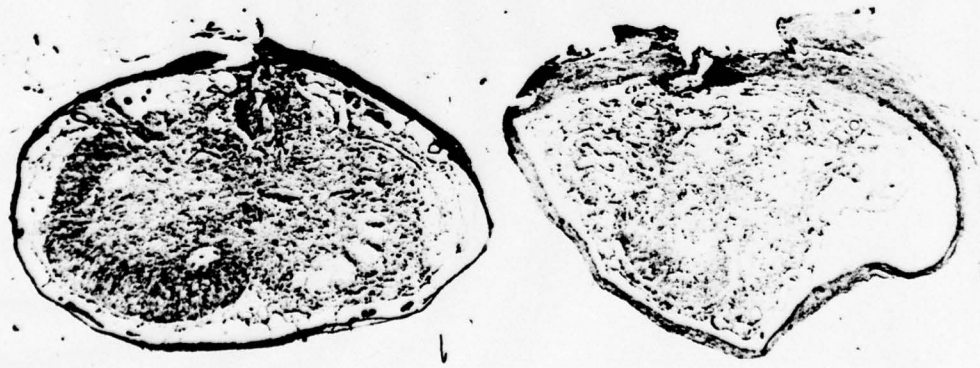

FIG. 3. Cat spinal cords at T-9. H & E, × 2. *Left:* At 6 weeks after 200 gm-cm impact injury and ethanol infusion. *Right:* At 8 weeks after 200 gm-cm impact injury and ethanol infusion.

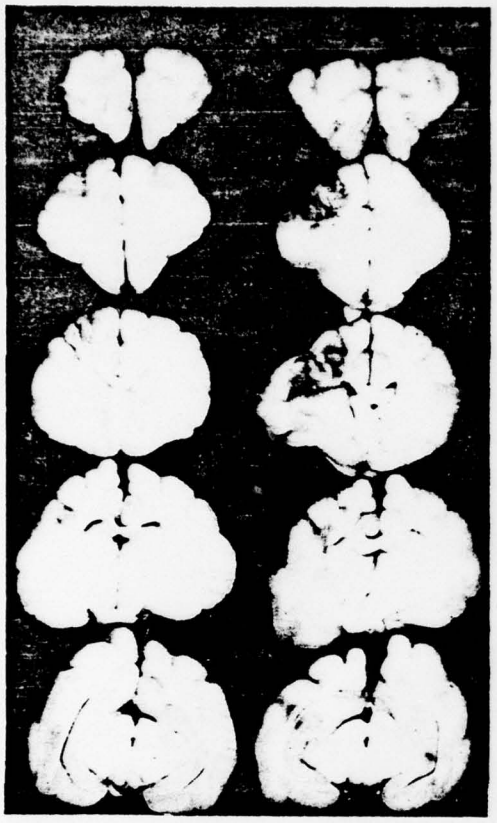

FIG. 4. *Left:* Coronal sections of brain 3 hours after 900 gm-cm impact injury. *Right:* Coronal sections of brain 3 hours after 900 gm-cm impact injury and ethanol infusion. The hemorrhage and extravasation of Evans blue are evident in the animal receiving ethanol.

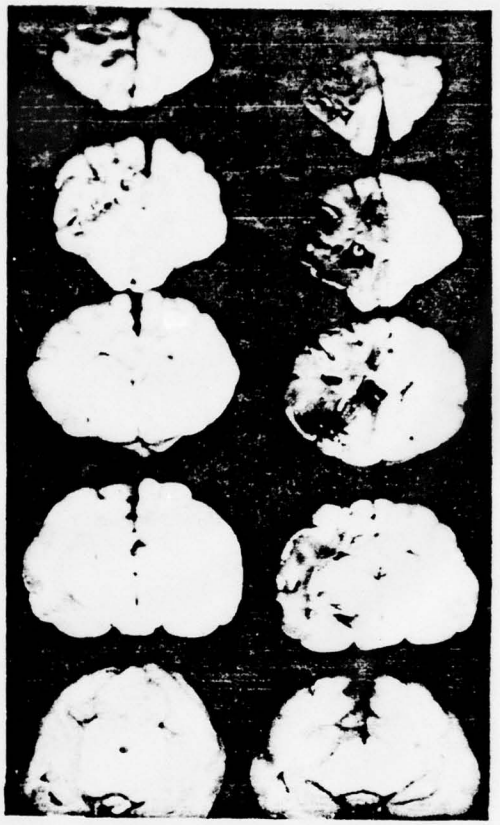

FIG. 5. *Left:* Coronal sections of brain 24 hours after 1000 gm-cm impact injury. *Right:* Coronal sections of brain 24 hours after 1000 gm-cm impact injury and ethanol infusion. The extravasation of Evans blue is seen in all sections of the animal receiving ethanol, but in only two sections of the control animal.

some extravasation of Evans blue in the white matter beneath the injury. However, in those animals that received ethanol before injury, the spread of Evans blue was two to three times more extensive (Fig. 4). The same phenomenon was observed in the group of animals that received a 1000 gm-cm impact. Ten animals were studied at 3 hours and nine at 24 hours. The lesions were more extensive in both the alcohol- and nonalcohol-treated groups than in the 900 gm-cm injury (Fig. 5). The same difference between alcohol- and nonalcohol-treated animals was observed. The area of extravasation of the Evans blue was three to four times as extensive in the ethanol animals as in the controls.

Histological studies of the brains confirmed the findings of the gross observations. An area of hemorrhage surrounded by edema corresponded to the area demarcated by the Evans blue.

Discussion

In both the spinal cord and brain studies, animals that received ethanol prior to their injuries sustained more extensive damage to the CNS than did identically injured control animals.

In the spinal cord injury model, a 200 gm-cm impact failed to produce significant deficits in the evoked potential, in acute

studies or in long-term investigations. However, in cats pretreated with ethanol, this same injury resulted in almost total impairment of the evoked potential, acutely, and in long-term studies caused paraplegia. The absence of evoked potentials for 3 hours post-injury has been a reliable criterion for predicting permanent paraplegia when using a 400 gm-cm impact force,[3] and proved to be an equally predictive parameter in the present studies.

The histological changes in spinal cords of animals receiving alcohol before injury paralleled the type of hemorrhagic necrotic lesions seen when a 400 gm-cm impact force is used.[2,15] The development of edema throughout the long tracts followed the central gray lesions, as in the 400 gm-cm impact model. Minor, or no histological changes, were seen in the injured controls.

In the cerebral contusion model, animals in both groups behaved similarly and, in fact, showed little in the way of specific neurological deficit 24 hours after their injury. This was in striking contrast to the wider zones of hemorrhagic necrosis and edema seen in the alcohol-treated cats, compared to those receiving the cerebral impact alone.

The observed increase in spinal cord and brain injury following ethanol administration is attributable to one or more of the known chemical and molecular effects of alcohol. If the factors responsible for this potentiation were known, some insight might be gained into the molecular mechanisms in the pathogenesis of CNS necrosis when larger injuries are delivered without alcohol. The blood levels of ethanol observed at the time of injury (450 mg% after 60 minutes of infusion) would be sufficient to produce severe intoxication in man.[28] Nevertheless, no change in evoked potential, neurological function, or histological appearance of the spinal cord was observed in those animals that received ethanol without any injury.

The more extensive hemorrhage in the CNS with alcohol and trauma may be a reflection of altered platelet function that has been reported after acute dosage of alcohol.[5,19] Platelet aggregation depends on a regulated production of prostaglandins from polyunsaturated lipids within platelet membranes. This process takes place via normal radical reactions,[16,17] and may be susceptible to perturbation by the membrane solubility of ethanol, as well as by the pathological radical reactions that can be initiated by ethyl alcohol.

The administration of alcohol or acetaldehyde is followed by a number of effects that are characteristic of adrenergic responses.[13,34] These responses may change the vascular tone and, combined with altered platelet function, may account for the development of increased hemorrhagic lesions in the impact-injured animals that were treated with alcohol. Other studies have demonstrated that acetaldehyde may result in disturbances of mitochondrial activity in brain and other organs.[4,14,18,29,30]

The most immediate effect seen after spinal cord injury is the change in the evoked potential in the animals treated with alcohol. This can hardly be explained by alterations in bleeding or clotting, since the changes occur within 1 to 2 minutes of the injury, at a time when no histological evidence of hemorrhage is present.[15] The kinetics of the hemorrhagic necrosis in the central gray, and the subsequent edema of the long tracts takes place slowly over a period of 6 hours, and is maximal at 24 hours. The most immediate effect of the usual paraplegia-producing injury of 400 gm-cm is the loss of ability to develop an evoked potential.[3] In the case of a 200 gm-cm impact, loss of evoked potential is rarely seen, unless the animal has been pretreated with ethanol. One of the implications of such a rapid loss of evoked potential after injury is that the mechanism for conduction along the cord is interrupted. Since this occurs at a time when no histological changes are evident, it may well be due to a change in the membrane mechanisms for conduction. The integrity of the membrane-bound enzyme, Na^+K^+-dependent ATPase, is essential for maintenance of appropriate ion transport to permit transmission of an impulse. For Na^+K^+-dependent ATPase to remain functional, fluidity of the long-chain fatty acid moieties of the membrane phospholipids must be maintained.[21] Free radical damage to membranes has been shown to inhibit Na^+K^+-ATPase activity, particularly via the additive or synergistic effects of trauma-induced radicals.[1] Numerous *in vitro* studies have demonstrated the detrimental effects of ethanol on Na^+K^+-ATPase,[20,32] and has been proposed as a basis for the depressant effect of ethanol on excitable membranes. It should be noted that in the present studies, the dose of ethanol was not sufficient to abolish the

evoked potentials without the addition of trauma, even though high blood levels were recorded. Thus, the combination of ethanol and trauma produces an effect on neural conduction, additive or synergistic, not seen with either variable alone.

Free radical lipid damage has been observed in a variety of settings both in severe (400 gm-cm impact) trauma to the central nervous system, as well as following exposure of hepatocytes to alcohol.[11,12,23] Ethanol, or its metabolite, acetaldehyde, can contribute to these membrane perturbations by adding directly across double bonds of the unsaturated fatty acids. This is a free radical addition and can be carried out by alcohols, aldehydes, and ketones, and thus result in significant structural and functional changes in cell membranes.[33] These reactions have been seen in hepatocytes and have been prevented by the addition of known antioxidants.[8,10-12,22] In cold-injured cerebral edema models, evidence has been obtained that indicates that radical reactions are involved since antioxidants offer protection, and also because radical end-products have been found.[25]

Although it is difficult and inappropriate to make correlations between these data and clinical problems seen in patients who have sustained trauma to the central nervous system after the ingestion of ethanol, we do feel that the use of these models may help to elucidate the molecular events that occur in biomembranes after trauma to the brain or spinal cord. Information at this basic level will suggest possibilities for preventive and therapeutic approaches.

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Spectrofluorescent Detection of Malonaldehyde as a Measure of Lipid Free Radical Damage in Response to Ethanol Potentiation of Spinal Cord Trauma

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ABSTRACT

Studies of the role of free radical damage to the spinal cord following a 400 g-cm impact have suggested an increase in at least one free radical product, malonaldehyde, 24-36 hr post injury. To investigate further the role of free radical lipid peroxidation in degeneration of the spinal cord following injury, a study of specific lipid fluorescence (SLF) indicative of the double Schiff-base adduct formed by a reaction between malonaldehyde and cellular components was carried out in the presence of ethanol, a known potentiator of free radical lipid peroxidation. The study was carried out in cats who received a 200 g-cm impact 3 hr to 23 days prior to sacrifice. Half of the impacted animals received ethanol, 5 ml/kg, prior to injury. These animals were rendered paraplegic, whereas the non-ethanol treated animals were neurologically intact. Controls consisting of laminectomies alone or laminectomies with ethanol but without injury were also studied. Spinal cord segments at the impact or laminectomy site were minced and extracted with chloroform-methanol, cleared by centrifugation, and examined in a scanning fluorometer with excitation maximum at 360 nm and emission maxima at 420, 440, 450, and 460 nm. SLF was minimal in cats 3 hr and 1 day post injury, but markedly increased at 3 days. By 5 days, background levels were again found in all groups. SLF in the alcohol-pretreated impact animals rose to a peak at 7 days, followed by a decline to background by 10 days. The presence of SLF supports a role for free radical lipid peroxidation in the degenerative changes in the spinal cord following injury. The findings of two peaks of SLF activity suggest two different sites of damage. One site, found acutely after injury, appears in all groups and was associated with reversible changes, while the other site is associated with later changes and chronic paraplegia only. The two sites could be the gray and white matter.

Pathologic free radical reactions among susceptible membrane lipids of the central nervous system following trauma have been suggested as a molecular basis for injuries to the central nervous system (1-3). Malonaldehyde, a product of free radical degradation of unsaturated fatty acids, has been shown to be elevated in spinal cords of cats injured with a 400 g-cm impact (4), as well as in freeze-lesioned rat brains (5). Other evidence suggesting that radical reactions may be involved in central nervous system injury include the consumption of endogenous tissue antioxidants following similar spinal cord injuries (3), the reduction of cerebral edema following cold injury by the administration of an exogenous antioxidant (6), reduction in malonaldehyde levels in freeze-lesioned rat brains with corticosteroid pretreatment (5), and the loss of extractable cholesterol from the central nervous system following cold injury (7).

A more recent model of spinal cord injury has been developed in this laboratory (8). It utilizes a 200 g-cm impact to the spinal cord of cats in conjunction with the systemic administration of ethanol, an agent known to enhance lipid free radical reactions. Animals injured with a 200 g-cm impact alone regain neurologic function, while those injured after the administration of ethanol remain permanently paraplegic. The present study was directed to the question of whether this physiochemical model of paraplegia might involve free radical mechanisms that could be measured.

Malonaldehyde is well known as a by-product of free radical peroxidation of polyunsaturated fatty acids (9). The most common method for measuring this 3 carbon dialdehyde utilizes its reaction with 2-thiobarbituric acid (TBA). Malonaldehyde, as produced, is not stable but a reactive molecule which is readily metabolized. It can also form cross-links with

TABLE I

Spinal Cord Injury – TBA Assay

Procedure	Time of sacrifice				
	0 hr	3 hr	5 hr	24 hr	Total
Laminectomy	4	2	2	2	10
Ethanol-laminectomy[a]	--	2	1	1	4
200 g-cm impact	--	4	3	3	10
Ethanol-200 g-cm impact[a]	--	4	3	3	10
Total	4	12	9	9	34

[a]Ethanol administered as an infusion of 95% ethanol, 5 ml/kg, diluted with saline to a volume of 100 ml, as a constant infusion for 1 hr prior to laminectomy or injury.

TABLE II

Spinal Cord Injury – Fluorescence

Procedure	Time of sacrifice							
	3 hr	24 hr	3 days	5 days	7 days	10 days	23 days	Total
Laminectomy[a]	4	4	7	6	9	8	4	42
200 g-cm impact	4	4	7	6	9	6	4	40
Ethanol-200 g-cm impact[b]	4	4	7	6	9	6	4	40
Total	12	12	21	18	27	20	12	122

[a]Laminectomy-ethanol animals (6) were sacrificed at critical times (3 hr, 3 days, 5 days) and found to have similar values to laminectomy alone. Thereafter all laminectomies were performed without prior infusion with ethanol.

[b]Ethanol administered as an infusion of 95% ethanol, 5 ml/kg, diluted with saline to a volume of 100 ml, as a constant infusion for 1 hr prior to laminectomy or injury.

amino-containing phospholipids, proteins, and nucleic acids (10). However, with these reactions, it becomes undetectable by the TBA test. These cross-links are conjugated double Schiff's base adducts and are fluorescent. They can be measured more reliably as an indicator of malonaldehyde production (10). In an attempt to avoid the inconsistencies observed with the TBA analysis, we have chosen to measure lipid soluble fluorescence (LSF) as an indicator of free radical reaction (11). In the present study, lipid soluble fluorescence and TBA-reactive material were measured in extracts of the traumatized spinal cords of cats which received or did not receive ethanol.

MATERIALS AND METHODS

Spinal Cord Injury

One hundred and fifty-six cats were used for the spinal cord injury study, 34 in the TBA study and 122 in the LSF study. All animals were anesthetized with sodium pentobarbital, 30 mg/kg. They were allowed to breathe spontaneously. Body temperature was maintained with a heating blanket. Arterial blood pressure and blood gases were monitored (8). Blood alcohol levels were measured spectrophotometrically with a dichromate assay (8).

Spinal cord injury was produced according to a method previously reported from this laboratory (8). A laminectomy was carried out from T7 through T11. A 20 g weight was dropped 10 cm down a Teflon tube to strike an impactor, thereby delivering a 200 g-cm force injury. Cortical evoked potentials were monitored prior to laminectomy, after laminectomy, and for 3 hr after injury.

A laminectomy group and a 200 g-cm injury group received 95% ethanol (5 ml/kg) diluted with saline to a volume of 30 ml as a constant infusion for 1 hour prior to laminectomy or injury. Fifty-two animals underwent laminectomy alone, four received ethanol prior to laminectomy alone; 50 animals received ethanol followed by a 200 g-cm impact; and 50 animals received a 200 g-cm impact without ethanol. Table I shows the distribution of the 34 cats used in the TBA assay study, and Table II shows the distribution of the 122 cats used in the LSF study, as well as the times of sacrifice of the different animals. After sacrifice, a 3 cm segment of spinal cord, centered over the site of injury, was removed for fluorescent analysis and TBA assay

TBA Assay

About 10% of the malonaldehyde found is free and produced following the oxidation of polyenoic fatty acids by double β-scission. This produces the 3-carbon dialdehyde and two hydrocarbon radical fragments. However, 90% of the oxidized fatty acids are meta-stable in the form of hydroperoxy- and cyclic endoperoxides (9). These meta-stable products are cleaved under the strongly acid conditions of the assay to form free malonaldehyde which is detected by the production of a chromogen, formed by reaction with two molecules of 2-thiobarbituric acid (TBA)(9). For the TBA assay, a weighed sample of spinal cord (about 0.4 g) was minced and homogenized in 5 ml of a mixture made up as follows: 10 ml of a saturated TBA solution in 10% perchloric acid (PCA) added to 30 ml of 20% trichloroacetic acid (TCA). The resulting homogenate was heated in a boiling water bath for 20 min and then centrifuged for 15 min at 2500 rpm. A 3 ml aliquot of the supernatant was removed and read at 532 nm on a Beckman DU spectrophotometer against a reagent blank.

Lipid Soluble Fluorescence

Spinal cord segments (1 g) were minced with two scalpel blades to less than 125 mm^3 pieces in 10 ml of carbonyl-free fluorescent grade chloroform and methanol (2:1, v/v) for several minutes, mixed with an equal volume of deionized water, and centrifuged for 5 min at 2500 rpm. One milliliter of the chloroform layer was mixed with 0.1 ml of methanol and exposed to high intensity ultraviolet light for 1 min (11). Fluorescence intensity was measured from excitation (360 nm) and emission (440 nm) spectra obtained at 25 C on a Hitachi-Perkin Elmer MPF 3 spectrofluorometer in the direct mode standardized with quinine sulfate (11). The measured wavelength was not corrected for internal instrument distortion. Excitation and emission slits were set at 8 nm; the sensitivity was set at 30. Fluorescence was recorded and expressed in arbitrary units based on these instrument settings. Typical fluorescent excitation and emission spectra are represented in Figure 1 (11). Fluorescence variation was generally not more than 20%.

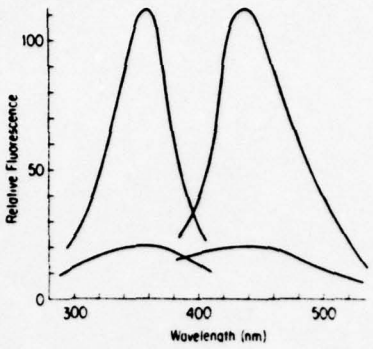

FIG. 1. Fluorescence excitation and emission spectra of chloroform:isopropanol extracts of tissue lipids. Maxima were 360 nm for excitation and 440 nm for emission. Lower curves were obtained from control tissue and show no distinct peaks, while upper curves are from a damaged tissue sample.

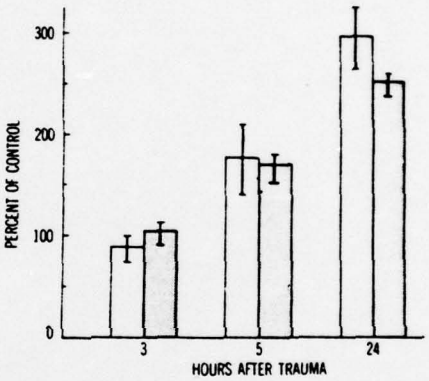

FIG. 2. Effects of alcohol and trauma on malonaldehyde levels in cat spinal cord. The ordinate represents a comparison of the means as a percent of the control (laminectomy alone). Vertical bars are the standard error of the mean (SEM). Although there was no statistical difference at any one time interval between the impacts and alcohol-impacts, there did appear to be significant differences between time intervals (see Table II). Open bars represent the 200 g-cm impact alone, closed bars represent alcohol-impacts. There are four animals in each 3 hr group, six in the 5 hr group, and six in each 24 hr group.

RESULTS

Spinal Cord Injury

During the 3-hr monitoring period, all 35 cats in the alcohol-laminectomy group retained their evoked potentials and were without neurological deficit after surgery. Those followed up to 23 days showed no neurological deterioration. The 37 animals in the 200 g-cm group had all regained their evoked potentials by the end of the 3-hr monitoring period and were walking by 7 days. The 37 animals in the alcohol-impact group lost their evoked potentials and did not regain them within the 3-hr monitoring period. These animals remained paraplegic until sacrifice (Table I).

TABLE III

Effects of Alcohol and Trauma on Malonaldehyde Levels in Cat Spinal Cord

Time (hours)	Malonaldehyde level[a] nmol/g	% of control	Significance (degrees of freedom, t value, P value)
3	31.2 ± 2.7	95.6 ± 8.4	
			t_{10}, 4.04, <.01
5	272.4 ± 27.1	173.0 ± 17.2	
			t_{10}, 4.08, <.01
24	340.2 ± 21.8	273.5 ± 17.5	

[a]Malonaldehyde levels by the TBA method were obtained by spectrophotometry and converted to nanomoles per g wet weight of tissue. The data were then converted to percent of laminectomy control for tabulation. Statistical analysis was performed using the Student's t-test, a comparison of the means. Confidence levels were chosen according to the two-tailed Z method. Since the impact groups and alcohol-impact groups showed no significant differences (Fig. 2), the two groups were pooled for each time and the combined groups compared against time. Since 5 hr animals were significantly higher than the 3 hr group, and the 24 hr animals also significantly higher than the 5 hr group, it follows that the 24 hr group was very significantly higher than the 3 hr group.

Malonaldehyde—Direct

At 3 hr, no differences in TBA reactive materials were observed between the impact group or the alcohol-impact group as compared to the controls (Fig. 2). At 5 and 24 hr, impacts and alcohol-impacts were two and three times the controls, respectively ($p<0.01$). Data from both groups were pooled for each time interval to perform Student's t-test for comparison of the means (Table III).

Malonaldehyde—Indirect

No differences were observed in the levels of lipid soluble fluorescence among the three groups when measured at 3 and 24 hr following sacrifice. A threefold increase was observed among all three groups at 3 days. By 5 days, this returned to background. A second elevation was observed at 7 days in the alcohol-impact group, but not in the laminectomy-ethanol group or the impact group. At 10 days and 23 days, lipid soluble fluorescence in all three groups had returned to background. These results are summarized in Figure 3.

Blood pressure and arterial blood gases did not vary significantly from baseline levels in all groups. The infusion rate of alcohol was adjusted to insure that bradycardia and hypotension did not occur. A mean blood level of ethanol of 350 mg % was obtained in the ethanol infusion groups. In the noninfused group, a blood level of 30 mg % ethanol was observed due to the ethanol contained in the commercial sodium pentobarbital preparation.

DISCUSSION

In the cat, a 400 g-cm impact to the exposed spinal cord alone invariably produces paraplegia (8). On the other hand, a 200 g-cm force produces a reversible lesion, unless the animals have been pretreated with ethanol to produce blood levels of 300-400 mg %, in which case, the same 200 g-cm force produces irreversible paraplegia (8). Malonaldehyde, a product of free radical peroxidation of lipids was measured in two ways: directly by the TBA assay and indirectly by lipid soluble fluorescence (LSF).

Malonaldehyde levels measured by the TBA assay rose in all three groups at 5 and 24 hr. However, no differences could be detected between impacted animals and animals pretreated with alcohol before impaction utilizing this assay (Fig. 2). TBA reactive material was not measured beyond the 24-hr period. LSF rose to similar levels above background at 3 days in all groups. By 5 days, LSF had returned to background in all groups. At 7 days, a second peak of LSF activity had occurred in the alcohol-impact group only. Only background LSF activity was noted after 7 days.

The source of malonaldehyde detected by both methods is most likely the polyunsaturated fatty acids of membrane phopholipids. Malonaldehyde cannot be produced as a consequence of radical damage to cholesterol or saturated fatty acids alone (12). Polyunsaturated fatty acids are needed (9). Phospholipids, as they occur in myelin and other cellular membranes, represent the largest source of unsaturated fatty acids in the central nervous system (13).

While LSF develops slowly and accumulates in detectable quantites only after several days, it is not entirely stable, since background levels are again achieved within 2 days after peak levels are produced. The induction period prior

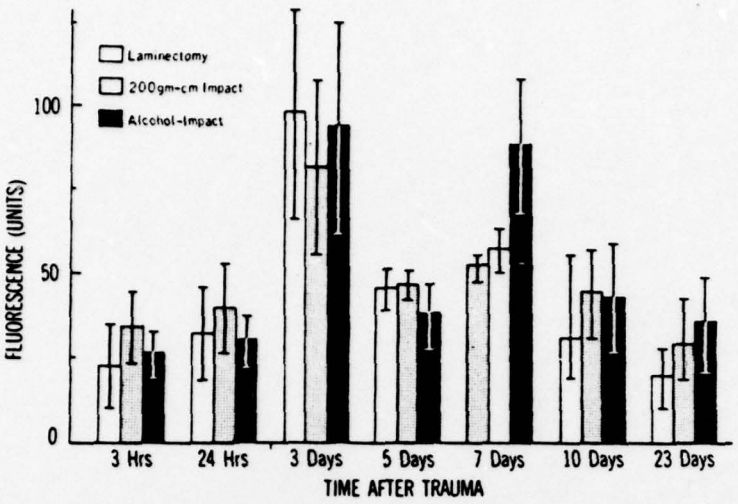

FIG. 3. Effects of alcohol on levels of lipid soluble fluorescence in traumatized spinal cord. The ordinate represents arbitrary fluorescence units taken from the photomultiplier reading and normalized to a constant setting. The open bars represent the laminectomy group, the stippled bars represent the 200 g-cm impact group alone, and the closed bars represent the alcohol-impact group. The vertical bars represent the SEM.

to the first fluorescence peak is due to single Schiff's base adducts which form rapidly from malonaldehyde in the presence of free amino groups. These do not contain conjugated double bonds and are not fluorescent (latent fluorescence) (10). Only when the second adduct is formed as a cross-link does the malonaldehyde product become fluorescent (expressed fluorescence) (10).

Although 1-2 days may be required for latent fluorescence from malonaldehyde to be expressed, once formed, it will be expressed (11). Of note, a second LSF peak occurs at 7 days, but only in the alcohol-impact group which developed paraplegia. Because of the kinetics of latent fluorescence, i.e., formation of the conjugated cross-link, this second peak is indicative of a second wave of malonaldehyde production (10). This suggests that the first peak of fluorescence, seen in all groups, is associated with acute, reversible damage related to the surgery and trauma, whereas the second peak is associated only with irreversible degeneration within the spinal cord.

The data are compatible with the hypothesis that free radical mechanisms are involved in the alcohol-impact injury model. Possibly the added burden of free radical reactions caused by the presence of alcohol depletes aqueous antioxidants such as ascorbate (3) to levels which allow irreversible membrane lipid damage. The two peaks of LSF activity seem to reflect two separate sources or pools of unsaturated fatty acids. The former one, which is rapidly oxidized, is not associated with any noticeable permanent deficit. The latter source, one which oxidizes at a slower rate (because of lower levels of polyunsaturates and a larger lipid-soluble antioxidant pool), is correlated with irreversible damage. The two different sources, for example, could be the central gray matter and surrounding long tracts.

The central gray area, whose phospholipids are largely polyunsaturated, would undergo peroxidation more easily as a result of minimal surgical trauma, such as laminectomy alone and impact alone. Necrosis of a thoracic segment of central gray tissue, for example, while producing local loss of some neuronal elements, produces no grossly discernible sequella like paraplegia. An early peak in malonaldehyde levels would be reached in both surgically treated groups of animals irrespective of alcohol pretreatment. Since a full complement of protective enzymes (e.g., superoxide dismutases, catalases, peroxidases, glutathione reductase and oxidase, ascorbate oxidase) exist in all actively metabolic cells of the CNS, the biochemical debris would be rapidly delimited and removed (14), although any physiological loss of function would not be reestablished. The early peak of malonaldehyde in the laminectomy and alcohol-laminectomy controls (by either TBA or LSF) would have to be the result of damage at the subcellular level, since histologically there is no discernible damage to central gray or white matter in these animals.

Quite the opposite is true of the white

matter in long tracts where edema accumulates. The kinetics of peroxidation in this highly saturated, cholesterol-rich (75% of total CNS cholesterol is found in the myelin) tissue is very slow. However, the myelin is largely metabolically inert. There are essentially no enzyme systems to bear the brunt of a peroxidative attack (14). Once the cholesterol has been oxidized to any appreciable extent, membrane integrity is quickly destroyed (15). Damage in this tissue would be irreversible.

Cholesterol in the plasma membranes of most cells represents a pool which is rapidly exchangeable with free plasma cholesterol (16). The opposite case exists for cholesterol contained in lipid droplets, organelle membranes within the cell, or the multilaminar extruded plasma membranes of the myelin sheath. Exchange rates of the order of 10% of total or less in 7 hr is a typical figure for lipid droplets and subcellular organelle membranes (16). Myelin, because of its multilayered configuration, and isolation from the general circulation, represents a terminal storage site for cholesterol (16). There are no mechanisms for removal of any damaged steroids from this site, other than by simple diffusion out of the membrane (16).

The data are generally supportive of the hypothesis that free radical mechanisms are operative during traumatic injury to spinal cord. Of even greater importance are the findings which suggest two major sites of damage in such injuries: one rapid and reversible, the other slow but permanent. If the two sites responsible for the production of malonaldehyde are, in fact, the central gray and surrounding white matter, then this represents a critically important concept for all of the CNS since, in any potentially reversible condition, it will be the white matter integrity which defines the limits of permanent damage.

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EFFECT OF TRAUMATIC STRESS ON CATECHOLAMINE SYNTHESIS AND METABOLISM IN THE SPINAL CORD

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In the past few years considerable attention has been focused on the role of biogenic amines in spinal cord injury. Elevated norepinephrine (NE) levels in traumatized spinal cords were implicated in the formation of the hemorrhagic necrosis (Ref. 1,2) or were thought to reflect the ingress of circulating amines (Ref. 3,4) as a result of disruption of the blood-spinal cord barrier rather than release from descending adrenergic fibers. In a previous communication (Ref. 5) we had found no change in endogenous NE concentrations but an increase in dopamine (DA) levels of the spinal cord one hour after contusion of the spinal cord. Other laboratories also have not found elevations of NE in similar experiments and after comparable degree of spinal cord trauma (Ref. 6,7). These findings contradicted the aforementioned results (Ref. 1-4). The present study was undertaken to resolve the controversy concerning the role of catecholamines in traumatized spinal cord.

Methods

Sixty immunized mongrel cats weighing from 2.5 to 3.0 kg were anesthesized by intravenous injection of pentobarbitol (30 mg/kg). All cats underwent laminectomy from thoracic seventh to eleventh (T7-T11) segments. L-tyrosine (2,6-^{3}H, specific activity 45 Ci/mM) was administered intravenously to three groups of cats (10 cats/group). One hour after injection of tyrosine (1 mCi/kg) each experimental animal received a 400 gm·cm impaction to the exposed dura at the ninth thoracic segment (T9). The animals were sacrificed one hour (Group I), two hours (Group II) and three hours (Group III) after contusion of the spinal cord.

Comparable to Group I another group of five cats (Group IV) were sacrificed one hour after impaction of the spinal cord or two hours after injection of the tritiated precursor. Group IV was injected intravenously with 0.5 ml/kg tritiated L-3,4-dihydroxyphenylalanine (^{3}H-L-dopa, ^{3}HG 6.5 Ci/m mole, New England Nuclear) approximately 45 minutes following the blockade of the peripheral aromatic L-amino acid decarboxylase (L-AAD) by intraperitoneal injection of MK-486* (25 mg/kg). Each experimental group had a corresponding laminectomy control group for the same period of time.

Arterial blood pressure was continuously monitored in all animals. The blood pressure was

*MK-486 = ℓ-α-(3,4-dihydroxybenzyl)-α-hydrazinopropionic acid monohydrate

permitted to stabilize for one hour before laminectomy and trauma. Averaged evoked cortical sensory action potentials (EP) were recorded from the region of the sigmoid gyrus during stimulation of the contralateral peroneal nerve before and after impact to ascertain the adequacy of the trauma (Ref. 5, 8).

A three cm portion of the spinal cord centered at the level of impact (T9) was removed in a temperature - controlled room (4°C) at the appropriate time after the injection of tritiated precursors. The specimens were divided into three (1 cm) equal parts. These spinal cord segments, labelled rostral, middle and caudal, were immediately frozen on dry ice or in liquid nitrogen and stored at -25°C until analyzed. The disappearance rate of ^{3}H-L-tyrosine and ^{3}H-L-dopa from the blood and the specific activities of DA and NE on each one cm segment of the spinal cord were determined.

Determination of Endogenous Biogenic Amines

The tissues were homogenized in acidified butanol. After centrifuging the homogenate, the supernatant containing catecholamines was shaken into water and adsorbed on alumina. Norepinephrine and dopamine were eluted from alumina and were assayed fluorometrically as described previously (Ref. 5).

Analysis of Labelled Dopamine and Norepinephrine

^{3}H-L-tyrosine and ^{3}H-L-dopamine and their metabolites ^{3}H-DA and ^{3}H-NE were analyzed as follows: each one cm segment of spinal cord was homogenized in acidified butanol, centrifuged, and the supernatant containing catecholamines was shaken into water and adsorbed on alumina as above. The eluate was adjusted to pH 2 and applied to a column of Dowex 50 W-X4 (200 - 400 mesh, 4mm x 2 cm). Norepinephrine and dopamine were eluted with 1 N HCl and 2 N HCl, respectively. Eluates containing NE or DA were quantitatively transferred to a counting cocktail (Aquasol, New England Nuclear) and the radioactivity was determined in a liquid scintillation spectrometer (Searle). The entire procedure was carried out in a temperature - controlled room (4°C).

L-Amino Acid Decarboxylase Activity

Spinal cord segment was homogenized in 5 volumes (w/v) ice cold 0.05 M Tris HCl buffer pH 6.0 containing 0.1% (v/v) Triton X-100 in a glass-Teflon homogenizer. The homogenate was centrifuged in a refrigerated centrifuge at 10,000 g for 10 minutes and the supernatant decanted for the enzyme assay (Ref. 9). An aliquot of the supernatant was incubated in a closed system with the carboxyl labelled substrate, L-3,4-dihydroxyphenylalanine (L-^{14}C dopa). The activity of L-AAD was determined by measuring the $^{14}CO_2$ product liberated by TCA and captured in plastic wells containing filter paper soaked in Hyamine; the entire well was then dropped into a scintillation vial containing Aquasol and counted in a liquid scintillation spectrometer. The units of L-AAD are given in nmoles CO_2/g wet tissue/hour.

Dopamine-β-Hydroxylase Activity

The spinal cord segment was homogenized and centrifuged similar to the method for L-AAD, except

that the Tris buffer was 0.005 M. The assay is based on β-hydroxylation of the substrate phenylethylamine by dopamine-β-hydroxylase (DβH) to phenylethanolamine (Ref. 10). The latter is converted to ^{14}C-labelled N-methylphenylethanolamine by purified bovine adrenal phenylethanolamine-N-methyltransferase in the presence of the active methyl donor s-adenosyl-methionine methyl-^{14}C. Dopamine-β-hydroxylase units are given in nmoles N-methylphenylethanolamine/g wet tissue/hour.

Monoamine Oxidase Activity

The spinal cord segments were homogenized in 10 volumes0.005 M phosphate buffer pH 7.0 and the enzyme activity was assayed on an aliquot of the homogenate. The method is based on the oxidative deamination of tritiated substrate ^{3}H-tyramine to tritiated product ^{3}H-p-hydroxyphenylacetic acid (Ref. 11). After separation of the product by extraction with ethyl acetate, the radioactivity was measured in a liquid scintillation spectrometer. Units are given in nmoles of p-hydroxyphenylacetic acid/g wet tissue/hour.

RESULTS

The circulating levels of labelled L-tyrosine and L-dopa dropped to 80% and 90% of the zero time concentrations within five and 10 minutes, respectively, after the administration of the labelled precursors. These findings are similar to those of Wurtman et al. (Ref. 12) using ^{14}C-L-dopa in the whole mouse. The circulating levels of total radioactivity reached a plateau 10 minutes after injection and remained fairly constant for three hours. There was no appreciable difference between the experimental and laminectomy control groups.

Effect of Trauma on the Biosynthesis of Dopamine and Norepinephrine

From ^{3}H-L-tyrosine precursor. There was no appreciable difference in concentrations of ^{3}H-NE and ^{3}H-DA among the three one cm segments of the spinal cord of the control groups, nor was there an appreciable variation among those of the experimental groups. Two hours after injection of ^{3}H-tyrosine the molar ratio of the tritiated DA to tritiated NE in the spinal cord of the control was approximately 1·3; from each fmole (mole x 10^{-15}) of DA about 0.8 fmole NE was synthesized (Fig. 1). The disappearance rate of ^{3}H-NE and ^{3}H-DA from the spinal cord was linear in the control groups. The decay curve of ^{3}H-NE was slower than that of ^{3}H-DA; the half-lives of ^{3}H-DA and ^{3}H-NE were about 3.5 and 4 hours, respectively. In control Group III the concentrations of ^{3}H-DA and ^{3}H-NE decreased to levels approximately 60% of those in control Group I (Fig. 1). One hour after spinal cord trauma the ^{3}H-DA concentration was 15% below that of control (Group I) but it had reached a plateau two hours after (Group II) and was 17% above that of the control three hours after impaction (Group III). One hour after trauma the ^{3}H-NE levels were 30% lower than those of the corresponding control (Group I) and remained relatively unchanged thereafter in all experimental groups (Fig. 1). One hour after impact the radioactive counts incorporated from ^{3}H-L-tyrosine into ^{3}H-DA were reduced from 5.4 in

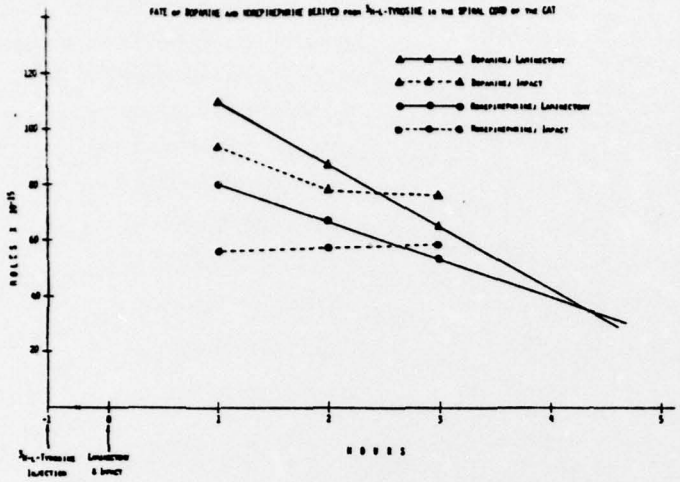

Fig. 1. L-tyrosine (2,6-^{3}H) was injected into three groups of cats which underwent laminectomy from T7-T11. One hour after intravenous injection of tyrosine each experimental animal received a 400 gm·cm impaction. The animals were sacrificed one hour (Group I), two hours (Group II) and three hours (Group III) after impaction of the spinal cord. Each experimental group had a corresponding laminectomy control group for the same period of time.

the control Group I to 4.4 nCi/g in the experimental Group I. Since after impact the endogenous DA concentrations were twice those of the control, the specific activity was sharply reduced after impact (Table 1). Radioactivity incorporated into ^{3}H-NE decreased from about 4.0 to 3.1 nCi/g. The endogenous NE concentrations, however, did not change appreciably. The specific activity of NE, therefore, was not significantly altered (Table 1).

From ^{3}H-L-dihydroxyphenylalanine precursor. In the spinal cord of the cats injected with ^{3}H-L-dopa the ratio of ^{3}H-DA/^{3}H-NE was 3·0 compared with the ratio of 1·3 in the comparable group of cats injected with ^{3}H-L-tyrosine. One hour after impact (Group IV) the average radioactive counts converted from ^{3}H-dopa to ^{3}H-DA were reduced; the incorporation of radioactivity into ^{3}H-DA fell from a mean of 20.4 in the control Group IV to 16.5 nCi/g in the experimental Group IV (Table 2). Endogenous levels of DA increased after trauma to levels approximately two-fold above that of the control. The mean specific activity of DA in spinal cord, therefore, decreased significantly one hour after impact (Table 2). The ^{3}H-NE in the impacted group (Group IV) was reduced compared with that of the laminectomy controls. Since the endogenous concentration of NE did not change appreciably

TABLE 1

Effect of Spinal Cord Trauma on the Biosynthesis of Dopamine and Norepinephrine From the Precursor ^{3}H-L-Tyrosine in Group I

	Dopamine		Norepinephrine	
	Laminectomy	Impact	Laminectomy	Impact
(A)	5.45 ± 0.79	4.38 ± 0.46	3.98 ± 0.57	3.08 ± 0.39
(B)	0.68 ± 0.09	1.48 ± 0.15*	0.89 ± 0.05	0.85 ± 0.05
(C)	7.98 ± 1.53	2.96 ± 0.43*	4.47 ± 0.68	3.62 ± 0.50

*P < 0.001

A = labelled, nCi/g; B = endogenous, nmole/g; C= specific activity, nCi/nmole

TABLE 2

Effect of Spinal Cord Trauma on the Biosynthesis of Dopamine and Norepinephrine From the Precursor ^{3}H-L-Dopa in Group IV

	Dopamine		Norepinephrine	
	Laminectomy	Impact	Laminectomy	Impact
(A)	20.42 ± 3.21	16.49 ± 1.46	7.11 ± 2.51	5.13 ± 1.37
(B)	0.68 ± 0.09	1.48 ± 0.15*	0.89 ± 0.05	9.85 ± 0.05
(C)	29.90 ± 6.02	11.14 ± 1.50*	7.99 ± 2.85	6.03 ± 1.63

*P < 0.001

A = labelled, nCi/g; B = endogenous, nmole/g; C = specific activity, nCi/nmole

after impact, specific activity of NE dropped but was not significant at 95% level (Table 2).

Metabolic enzymes. The activity of the enzymes in laminectomy control Group I and its corresponding experimental group (one hour after impaction of the spinal cord) are shown in Table 3. There was no appreciable change in the activities of L-AAD and DβH. In the impacted group the monoamine oxidase activity was significantly lower than that of the control.

TABLE 3

Effect of Spinal Cord Trauma on Activity of the Enzymes of Catecholamine Pathway

	L-AAD	DβH	MAO
Laminectomy	143 ± 12.6	26.5 ± 1.3	2048 ± 66
One hour after impact	127 ± 8.7	23.7 ± 2.1	1672 ± 58
P	N.S.	N.S.	<0.01

units of enzyme activity - nmoles/g wet tissue/hour

N.S. = not significant

Discussion

After trauma the amount of radioactivity incorporated from both ^{3}H-L-tyrosine and ^{3}H-L-dopa into ^{3}H-DA and ^{3}H-NE is sharply reduced when compared with that of laminectomy controls (Fig. 1, Tables 1 and 2). The rise in the levels of endogenous DA in all segments of the spinal cord in Group II cats may reflect a reduction in the activity of DβH which in the central nervous system synthesizes NE effectively only when membrane bound (Ref. 13). Trauma to the cord may alter this state and thereby reduce DβH activity. DA and NE, released from the storage vesicles after trauma, are subject to degradation by MAO and catechol-o-methyl transferase. The specific activity of DA in Groups II and IV was sharply lower than that of corresponding laminectomy control groups, due to a dilution of the labelled DA in a larger pool of endogenous DA accumulated after trauma because of impaired DβH activity. Monoamine oxidase, a membrane bound mitochondrial enzyme which catabolizes both DA and NE to their corresponding dihydroxy acid metabolites, cannot be implicated in reducing the amount of ^{3}H-DA, since it would also catabolize the endogenous DA comparably and the specific activity of DA would, therefore, remain unchanged.

Analysis of MAO activity *in vitro* demonstrates that one hour after impact activity of the enzyme is significantly reduced (Table 3). The reduction in specific activity of DA after contusion of the spinal cord also indicates that the activity of tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of NE (Ref. 14), is reduced. Since the incorporation of radioactivity into ^{3}H-DA is reduced using both precursors ^{3}H-tyrosine and ^{3}H-dopa it follows that the activity of L-AAD is also impaired. Table 3 shows that the activities of L-AAD and DβH, although lower after impact, were not significantly different from those of the control. The reason for this apparent paradox is that *in vivo*, the concentration of molecular oxygen, a primary substrate for tyrosine

hydroxylase, DβH and MAO is sharply reduced due to local circulatory stasis and anoxia. In vitro provided with all co-factors including oxygen, DβH activity in the spinal cord after impact remains relatively unchanged; within the time interval of the experiment little protein denaturation has taken place.

The finding of large amounts of endogenous DA in the spinal cord indicates that DA in the spinal cord does not serve only as a substrate for DβH but may rather be stored in specific dopaminergic storage pool(s) and possibly serve as an inhibitory neurotransmitter of internuncial neurons. If all DA in the spinal cord served only as a substrate for DβH rather than being stored in specific dopaminergic storage reserves, endogenous DA would be expected to be rapidly transformed to NE and stored in noradrenergic vesicles.

The results demonstrate that (1) not only tyrosine hydroxylase is present but also the whole complement of the NE synthesizing machinery exists in the spinal cord. As early as five minutes after adminstration of L-2',6'-ditritiotyrosine, both DA and NE are detected and 15 minutes after injection they are found in quantitative amounts in the spinal cord at the level of thoracic seventh to eleventh (T7-T11) dermatomes. The rate of axoplasmic flow, 0.7 mm/hour (Ref. 15) is too low to account for the formation of DA and NE in the brain stem neurons and subsequent transport to the T7-T11 level. (2) There is a reduction in the activity of L-AAD as evidenced by lower amounts of ^{3}H-DA formed from both ^{3}H-L-tyrosine and ^{3}H-L-dopa precursors. (3) The activity of membrane bound enzymes DβH and mitochondrial MAO is curtailed, probably due to circulatory stasis, hypoxia, and disruption of the membrane integrity. This condition results in accumulation of DA and no change in NE concentration with time after injury. (4) Since L-AAD is distributed ubiquitously and tyrosine hydroxylase is intraneuronally confined, it can be deduced that for every mole of NE formed from tyrosine, approximately two moles are formed extraneuronally when dopa is used as a precursor. (5) The restoration of impaired NE synthesizing machinery by membrane stabilizing drugs such as epsilon aminocaproic acid and methylprednisolone sodium succinate (Ref. 5) further suggests that the changes seen after trauma may be secondary to a more diffuse process of membrane dysfunction (Ref. 5, 16,17). (6) From these results it is difficult to support a direct etiologic role for catecholamines in the production of the pathophysiologic events seen after spinal cord injury.

Summary

Three groups of cats were injected with L-tyrosine (2,6,-^{3}H). All cats underwent laminectomy from T7-T11. One hour after intravenous injection of tyrosine each experimental animal received a 400 gm·cm impaction to the exposed dura. The animals were sacrificed one hour, two hours, and three hours after contusion of the spinal cord. Following blockade of peripheral L-AAD by administration of MK-486, L-^{3}H-dopa was also administered I.V. to a group of five cats which were sacrificed

one hour after impaction of the spinal cord. Each experimental group had a corresponding laminectomy control group for the same period of time. In the spinal cord of the control groups, the molar ratio of the tritiated DA to tritiated NE was approximately 1·3; from each fmole (mole x 10^{15}) of DA about 0.8 fmole NE was synthesized. In the control groups the disappearance rate of NE and DA from the spinal cord was linear; the half-lives of DA and NE were 3.5 and 4 hours, respectively. The disappearance rate of DA decreased one hour and plateaued two hours after impaction, compared with the control. One hour after spinal cord trauma the DA concentration was 15% below that of control but three hours after impaction DA concentration was 17% that of the control. After trauma the NE levels were approximately equal in all experimental groups. In the spinal cord of the cats injected with ^{3}H-L-dopa the ratio of DA/NE was 3·0 compared with the ratio of 1·3 in the cats injected with ^{3}H-L-tyrosine. The results demonstrate that (1) there is a reduction in the activity of tyrosine hydroxylase and/or L-AAD as evidenced by lower amounts of ^{3}H-DA formed from both ^{3}H-L-tyrosine and ^{3}H-L-dopa precursors; (2) the activity of membrane bound enzymes DβH and mitochondrial MAO is curtailed, probably due to circulatory stasis, hypoxia, and disruption of the membrane integrity. This condition results in accumulation of DA and no change in NE concentration with time; (3) since L-AAD is distributed ubiquitously and tyrosine hydroxylase is intraneuronally confined, it can be deduced that for every mole of NE formed from tyrosine, approximately two moles are formed extraneuronally when dopa is used as a precursor.

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DISCUSSION

Dr. Kopin inquired whether there was a change in the blood/spinal cord barrier as a result of the manipulators. Dr. Naftchi indicated there was not since there was very little ingress of radioactive DA or NE from the blood after impacting the cord.